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SCHOOL OF AVIATION MEDICINE
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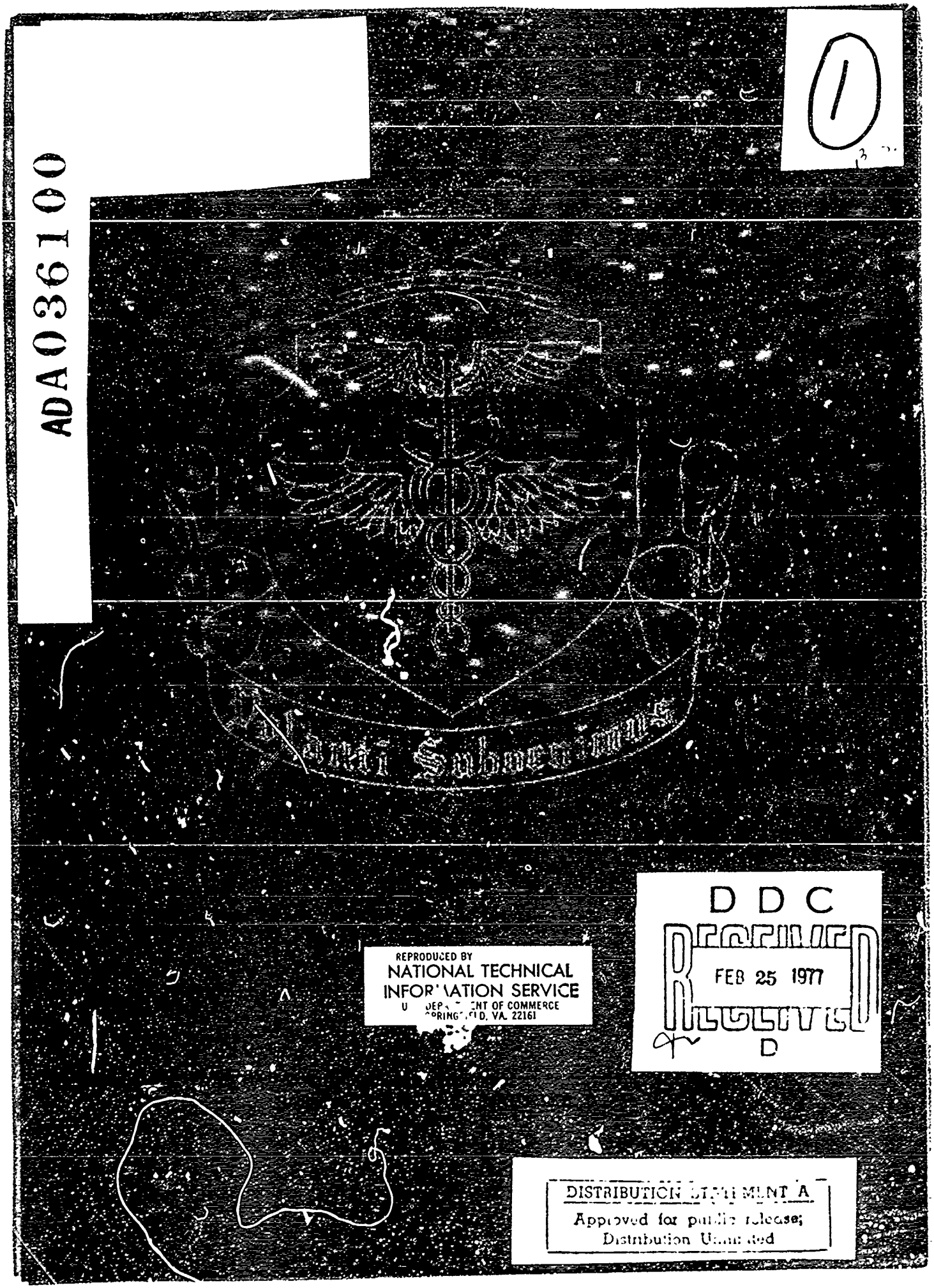

EFFECTS OF BETA-AMINOETHYLISOTHIOUREA (AET) ON ENZYMES OF ANIMAL TISSUES

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The effects of the radioprotective compound β-aminoethylisothiourea (AET) on sulfhydryl enzymes of mammalian tissues have been determined, in an effort to obtain information regarding the biologic activity of the drug. A number of enzyme systems involved in oxidative carbohydrate metabolism of rat tissues were inhibited in vitro by concentrations of AET within the predicted in vivo range following sublethal doses. Two of these enzyme systems, the pyruvic and alpha-ketoglutaric acid oxidizing systems of rat liver and kidney, were also markedly inhibited in vivo by AET. Inhibition was not due to an effect on coenzymes involved in the reactions but may have been caused by inactivation of the electron transport system. The time of onset of enzyme inhibition and the rate of reversal were in agreement with the onset and severity of toxic manifestations in the rat following AET administration and, furthermore, were correlated with the time of maximum radioprotection. However, a study of the susceptibility of several liver enzymes in different species failed to establish a relationship between either the toxicity or the radioprotective activity of AET and the degree of sulfhydryl enzyme inhibition. AET also stimulated anaerobic glycolysis in rat liver slices and produced lactacidemia in rats after parenteral administration. Efforts to locate the enzymatic site of the glycolytic action were not successful. The results of this study demonstrate the feasibility of a theory of radioprotective mechanisms involving the formation of radioresistant complexes between the protective agent and important biologic sulfhydryl groups.

Considerable attention is being given to a search for chemical agents which have prophylactic or therapeutic value against the toxic effects of ionizing radiations. The recent discovery by Doherty and Burnett (1) of the radioprotective activity of β-aminoethylisothiourea (AET) in mice has also been shown by Crouch and Overman (2) to be applicable to monkeys. Although AET is one of the most effective radioprotective agents known at present for certain species of experimental animals, the practical use of this compound in man would apparently be hazardous because of the marked toxic effects which it produces at relatively low dosage levels (3).

DiStefano et al. (4) have shown that AET exerts a wide variety of pharmacologic actions in cats, including a biphasic blood pressure response, inhibition of the nictitating membrane, apnea, and convulsions. The biochemical mechanism by which AET produces these toxic effects in aminals and man is unknown. According to Shapira et al. (5) AET undergoes rearrangement in neutral aqueous solutions to form mercaptoethylguanidine which posseses a free sulfhydryl group. The in vivo studies of Conte and Melville (6) and the in vitro studies of Shapiro (7) have shown that AET combines with protein constituents of serum. The former authors found that this combination consists of a reaction between sulfhydryl groups to form mixed disulfides. In view of these findings it seemed possible that some of the effects of AET in mammals might be due to an interaction with enzymes and other cellular constituents bearing essential sulfhydryl groups. The present study was undertaken to ascertain the effects of AET on enzyme systems in an effort to obtain information on the mechanisms which might be responsible for the toxic and radioprotective actions of this compound. Results of the study have shown that several reactions in which sulfhydryl enzymes participate are inhibited by AET.

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MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (200 to 300 gm.), Carworth Farms mice (20 to 30 gm.),

guinea pigs (500 to 800 gm.), and mongrel dogs were employed for these studies. They were maintained on commercial, unrestricted diets in air-conditioned quarters. Rats, mice, and guinea pigs were sacrificed by decapitation and exsanguination. Dogs were stunned with an electric shock before being exsanguinated. The dichloride salt of AET[1] was used in all experiments. Aqueous solutions of AET were freshly prepared and neutralized with 1 normal sodium hydroxide immediately prior to use. For in vivo experiments AET was administered by the intraperitoneal route.

Measurements of the endogenous respiration of slices of rat liver, spleen, and thymus glands were performed manometrically at 38° C. in an atmosphere of pure oxygen. Free-hand tissue slices were suspended in Krebs-Ringer phosphate buffer and oxygen consumption was measured for a period of 60 minutes. If substrates were added the final concentration was 0.02 molar. The manometric method of Pardee and Potter (8) was used to determine the effects of AET on the citric acid cycle and for these experiments the sodium salts of pyruvic and fumaric acids were added to the test system at a final concentration of 5.3×10^{-3} molar. This system was also employed for measuring the rate of oxidation of pyruvate by tissue homogenates but in this case activity was estimated on the basis of chemical analysis for substrate disappearance. Alpha-ketoglutaric acid oxidase activity was measured according to the procedure of Ackermann (9) with a substrate concentration of 5.3×10^{-3} molar. The quantities of tissue employed in studies on the citric acid cycle and on alpha-ketoglutaric acid oxidation were as follows: rat and mouse liver, 50 mg.; dog liver, 100 mg.; guinea pig liver, 200 mg.; rat kidney, 30 mg.; rat heart, 40 mg.; and rat brain, 50 mg.

The succinic dehydrogenase and cytochrome oxidase activities of liver from rats, mice, and dogs were assayed by the manometric method of Schneider and Potter (10) while the malic dehydrogenase activity of rat liver and kidney homogenates was determined by the procedure of Potter (11). The diphosphopyridine nucleotide hydrogenase (DPNH) - cytochrome *c* reductase activity of rat liver mitochondria was measured spectrophotometrically by following the disappearance of the absorption peak of reduced diphosphopyridine nucleotide (DPN) at 340 mμ. Mitochondria were prepared according to the method of Schneider (12). The reaction was carried out in silica curvettes in a Beckman DU spectrophotometer at 25° C. and the reaction mixture contained 0.05 molar sodium phosphate buffer (pH 7.4), 1.33×10^{-5} molar cytochrome *c*, and 400 μg. of DPNH (Sigma) in a final volume of 3 ml. Optical density changes were recorded at 30-second intervals for a period of 5 minutes, during which time the activity of mitochondria from 5 and 10 mg. of tissue remained constant and was directly dependent upon tissue concentration. Anaerobic glycolysis and the anaerobic conversion of glucose to lactic acid were measured manometrically and colorimetrically using rat liver slices suspended in 3 ml. of Krebs-Ringer-bicarbonate buffer in Warburg vessels. The reaction was carried out under an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide for 1 hour at 38° C.; glucose, when used, was added at a final concentration of 0.02 molar. Phosphorylase activity was measured in liver slices of well-fed rats, according to the method of Sutherland and DeDuve (13). The reaction was carried out in 20 ml. beakers in a metabolic shaker at 38° C.

The activity of crystalline yeast hexokinase (Pabst) was measured using the manometric method of Colowick and Kalckar (14) with an adenosine triphosphate concentration of 5.3×10^{-3} molar. The adenosine triphosphatase activity of mouse tissues was determined by the method of DuBois and Potter (15). Twice recrystallized lactic dehydrogenase from rabbit muscle (Sigma) was used to determine the effects of AET on the reversible reaction catalyzed by this enzyme. The forward or reductive reaction was studied using the method of Busch and Nair (16) while the procedure of Zelitch (17) was employed to study the reverse or oxidative reaction. Preincubation of the enzyme with AET in the study of both reactions was carried out in 0.1 molar potassium phosphate buffer (pH 7.4) in a water bath at 38° C.

The procedure of Potter (18) was used for measuring the effect of AET on the in vivo

[1]Synthesized in this laboratory by Stanley J. Brois.

synthesis of citric acid in rat tissues. For these experiments animals were given 200 mg./kg. of AET 15 minutes before the administration of 3.5 mg./kg. of sodium fluoroacetate. The excretion and acetylation of sulfanilamide by rats was measured according to the method of DuBois et al. (19). Measurements of blood lactate and pyruvate concentrations were carried out on trichloroacetic acid filtrates of oxalated whole blood obtained from rats by cardiac puncture under light ether anesthesia. The animals were fasted for 16 hours prior to withdrawal of zero-time samples.

The chemical determinations and the particular procedures used for them in this study were: pyruvate and alpha-ketoglutarate (20), acetoacetate (21), lactate (22), glucose (23), inorganic phosphorus (24), citrate (25), and sulfanilamide (26).

RESULTS

Effect of AET on endogenous respiration and substrate oxidation by rat tissue slices in vitro

The initial phase of this study was carried out on tissue slices to obtain information on the ability of AET to alter the oxidative metabolism of animal tissues. It was found that the addition of concentrations of AET ranging from 1×10^{-3} molar to 3×10^{-3} molar to slices of rat liver, spleen, and thymus gland in vitro produced no significant change in the rate of endogenous respiration of these tissues. However, when pyruvate and succinate were added to the medium a concentration of 3×10^{-3} molar AET produced 78 percent and 49 percent inhibition, respectively, of the oxidation of these substrates by liver slices. The addition of 0.02 molar pyruvate to spleen and thymus slices caused no stimulation of the oxygen consumption by these tissues but chemical measurements of substrate disappearance revealed that appreciable amounts of substrate were removed from the medium by the tissues. A concentration of 2×10^{-3} molar AET caused 73 percent stimulation of pyruvate utilization by thymus slices but no change was observed with spleen slices. The fate of the increased quantity of substrate utilized by thymus slices was not determined.

Influence of AET on the function of the citric acid cycle in rat tissues

To test the possibility that AET affects the citric acid cycle, experiments were conducted in which AET was employed at a final concentration of 2×10^{-3} molar with rat liver, kidney, heart, and brain homogenates in the test system of Pardee and Potter (8). For in vivo tests a dose of 300 mg./kg. of AET was administered intraperitoneally 30 minutes before removal of tissues for assay. The results of these experiments are summarized in figure 1. The in vitro effect of AET was most pronounced with kidney homogenates in which virtually complete inhibition was produced within a period of 30 minutes. In the remaining tissues the rate of oxidation was progressively depressed by AET until at the the end of a 40-minute reaction period 45 percent, 42 percent, and 39 percent inhibition was observed in liver, brain, and heart, respectively. However, AET did not produce this inhibitory effect when it was administered to intact animals.

Effect of AET on the oxidation of alpha-ketoglutaric acids by rat tissues

In order to obtain more definitive information on the susceptibility of citric acid cycle enzymes to inhibition by AET, measurements of the effects of the compound on alpha-ketoglutaric acid oxidation by homogenates of several rat tissues were performed. The results of these experiments are presented in table I. In the presence of 2×10^{-3} molar AET, pyruvate oxidation in liver and kidney was reduced 60 percent and 64 percent, respectively, from control activities of 5.2 and 5.8 μM. of substrate utilized/40 minutes. This concentration of AET caused 21 percent inhibition of pyruvate oxidation by cardiac muscle from a control level of 6.1 μM./40 minutes while brain was refractory to the action of the drug. Data obtained by determining the effects of a series of concentrations of AET showed that 50 percent inhibition of pyruvate oxidation is produced by 4.3×10^{-4} molar and 3.1×10^{-4} molar AET in liver and kidney, respectively. The action of AET on pyruvate oxidation by liver was also determined in the presence of 0.004 molar malonate which was shown by

Lehninger (27) to cause quantitative conversion of pyruvate to acetoacetate. Under these conditions 50 mg. of normal liver synthesized 2.0 μM. of acetoacetate in 40 minutes and this reaction was inhibited 50 percent by a concentration of 3.3×10^{-4} molar AET. These results suggest that the site of AET inhibition is the oxidative decarboxylation of pyruvate since the pathways leading to citrate and acetoacetate are inhibited to the same extent. For in vivo experiments, rats were given various doses of AET 30 minutes before the removal of tissues for assay. The data in table I indicate that doses of AET well below the toxic range of 300 to 350 mg./kg. markedly reduce the ability of rat liver and kidney to oxidize pyruvate.

The findings of Best et al. (28), which revealed a depression of acetylation reactions by AET in vitro, suggested a possible mechanism by which the drug causes the inhibition of pyruvate oxidation observed in our experiments. To test this possibility rats were given 200 mg./kg. of AET 15 minutes before the intraperitoneal administration of 100 mg./kg. of sulfanilamide and the urinary excretion of sulfanilamide and its acetylated derivative was measured on 24-hour urine samples. Control animals which received no AET excreted 79 percent of the total dose of sulfanilamide within a 24-hour period, 60 percent of which was acetylated, and identical values were obtained for the AET-treated animals. It can thus be concluded from these experiments that AET produces no significant effect on the function of coenzyme A in vivo.

The in vitro and in vivo effects of AET on alpha-ketoglutarate oxidation by rat liver are also presented in table I. Normal liver utilized substrate and consumed oxygen at the rate of 9.1 μM. and 5.3 μM./40 minutes, respectively. These rates were inhibited 67 percent and 66 percent, respectively, in the presence of 2×10^{-3} molar AET while a concentration of 6.6×10^{-4} molar AET brought about 50 percent inhibition of the reaction. A relatively high in vivo sensitivity was also revealed by the fact that a dose of AET as

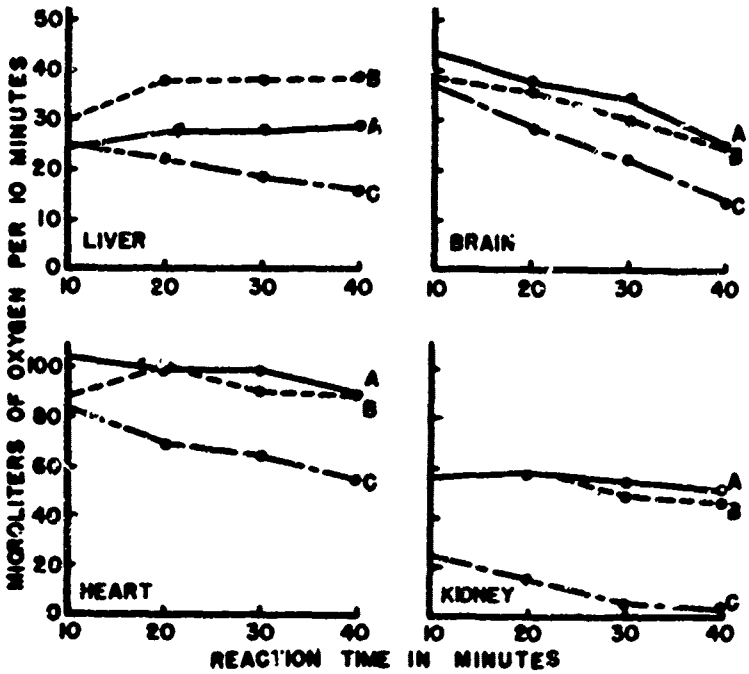

FIGURE 1

The effect of AET on the oxidation of pyruvate plus fumarate by rat liver, brain, heart, and kidney in vitro and in vivo; curve **A**, *normal control; curve* **B**, *30 minutes after 300 mg./kg. of AET intraperitoneally; curve* **C**, 2×10^{-3} *molar AET in vitro.*

TABLE 1

Effect of AET on alpha-ketoglutaric acid oxidation by rat tissues in vitro and in vivo

	Pyruvate oxidation			Alpha-ketoglutarate oxidation	
	Liver		Kidney	Liver	
	Substrate utilization	Acetoacetate formation	Substrate utilization	Substrate utilization	Oxygen consumption
	Average percentage inhibition in 4 animals				
2×10^{-3} molar AET in vitro	60	--	64	67	66
100 mg./kg. AET in vivo	23	25	9	23	–
200 mg./kg. AET in vivo	50	45	57	25	–
300 mg./kg. AET in vivo	71	69	71	41	32

low as 100 mg./kg. caused 23 percent inhibition of enzyme activity. The addition of 50 μg. of diphosphopyridine nucleotide and thiamine pyrophosphate to the system failed to either enhance the normal activity or reverse the AET-induced inhibition in vitro and in vivo, thus indicating that the action of AET is not due to an effect on these coenzymes.

In our initial experiments it was found that an apparent oxidation of AET by rat tissues tended to mask enzyme inhibition when oxygen consumption was used as a means of determining enzyme activity. Further investigation of this possibility revealed that when AET at a concentration of 2×10^{-3} molar was incubated with 50 mg. of liver for 40 minutes in the test system of Pardee and Potter (8), 67 μl. of oxygen were consumed as compared to 31 μl. in the absence of AET. The increase in oxygen consumption was apparently due to oxidation of the drug. Additional tests using other tissues showed that kidney oxidized AET at a rate similar to that of liver while heart and brain were less active. These findings stimulated our interest in determining whether the oxidation product of AET was the inhibitor of alpha-ketoglutaric acid oxidation. For these experiments the percent of normal liver and kidney activity was determined at 10-minute intervals in the presence of 2×10^{-3} molar AET. The average results of three experiments are shown in figure 2. These data demonstrate a linear relationship between the inhibition of alpha-ketoglutaric acid oxidation by AET and the length of the reaction period, with almost complete inhibition occurring in 40 minutes. These findings indicate that AET must first undergo an oxidative change to become an inhibitor of these reactions.

In the event that sulfhydryl enzyme inhibition is associated with the toxicity of AET, it might be expected that reversal of enzyme inhibition would accompany the reversal of symptoms following the administration of sublethal doses of the compound whereas no reversal should occur following a lethal dose. To test this possibility AET was administered to rats at dosage levels of 190 mg./kg. and 380 mg./kg. and the livers from groups of 4 animals were assayed at subsequent intervals for alpha-ketoglutaric acid oxidizing activity. The results of these experiments are shown in figure 3. Maximum inhibition of enzyme activity occurred at 30 minutes after administration of 190 mg./kg. of AET but reversal to normal occurred within 3 hours. Following a dose of 380 mg./kg., which is above the LD_{50} of AET for rats (29), only moderate

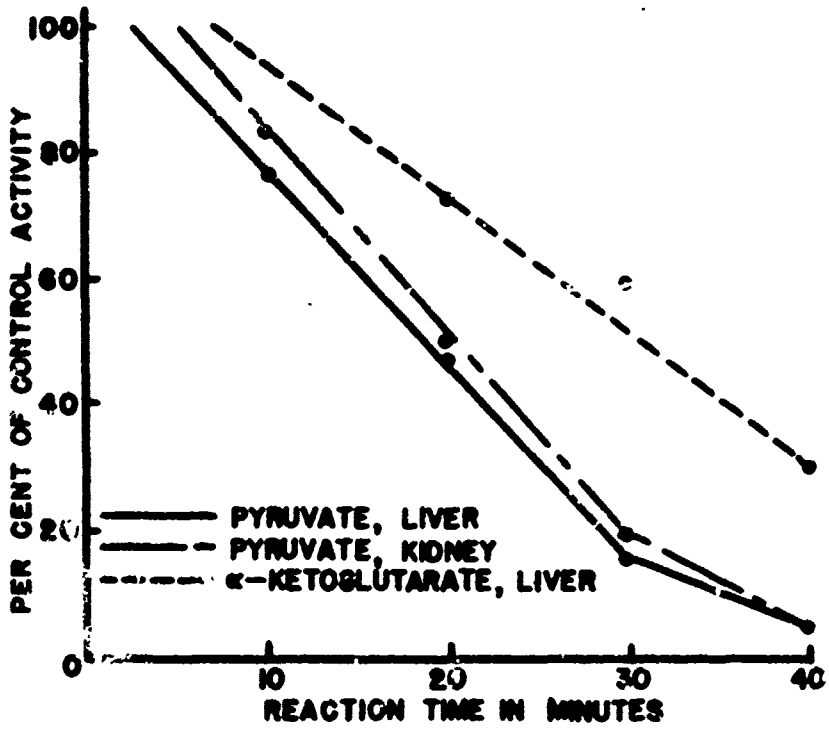

FIGURE 2

The effect of incubation time on the percentage of inhibition by AET of pyruvate and alpha-ketoglutarate oxidation by rat liver and pyruvate oxidation by rat kidney in vitro.

reversal of enzyme inhibition was noted at 3 hours. At this dosage level the animals may be expected to die within 2 to 6 hours.

Effect of AET on citrate accumulation in rat tissue

The procedure of Potter (18), which utilizes fluoroacetate to prevent the further metabolism of citrate, was employed to ascertain the effect of AET on citric acid formation in various rat tissues. It was found that a dose of 200 mg./kg. of AET administered 15 minutes prior to fluoroacetate injection caused 27 percent, 31 percent, and 26 percent reduction of citrate accumulation in liver, kidney, and heart, respectively. The control levels of citrate formation under the conditions of this experiment were 111, 1,403, and 577 μg./gm. of tissue for liver, kidney, and heart, respectively. The significance of the inhibition in liver is questionable in view of the low concentration of citrate in this tissue. No change was noted in the ability of spleen, thymus gland, or brain to accumulate citrate following administration of AET.

Effect of AET on various dehydrogenases of rat tissues

The marked inhibitory action of AET on alpha-ketoglutaric acid oxidation stimulated our interest in the effect of the compound on other dehydrogenases which require sulfhydryl groups for activity. It was found that a concentration of 1.2×10^{-3} molar AET produced 50 percent inhibition of the succinic dehydrogenase activity of rat liver (normal Q_{O_2} of 78). However, at 30 minutes after the intraperitoneal injection of 190 mg./kg. and 380 mg./kg. of AET, the succinic dehydrogenase activity was inhibited only 5 percent and 24 percent, respectively.

A study of the effects of AET on malic dehydrogenase activity revealed that the sensitivity of this enzyme was essentially equal to that of succinic dehydrogenase. The normal Q_{O_2} values for liver and kidney were 75 and 70, respectively. Fifty percent inhibition of liver enzyme activity was produced by a concentration of 2×10^{-3} molar AET. Livers from rats which

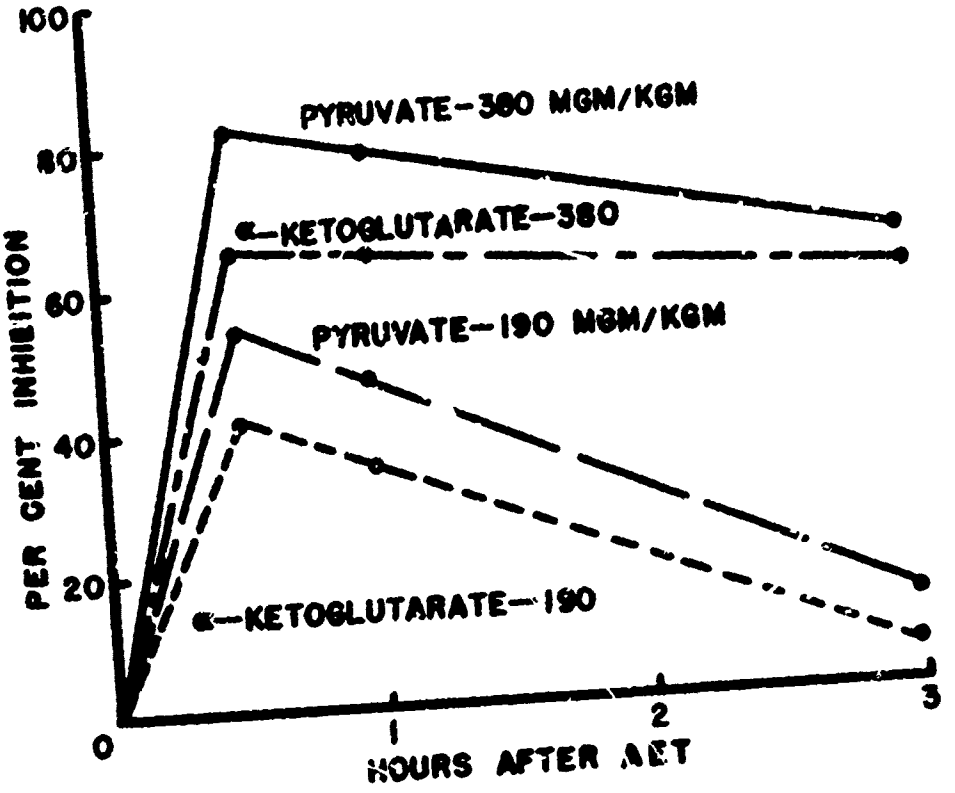

FIGURE 3

The effect of sublethal and lethal doses of AET on the duration of inhibition of pyruvate and alpha-ketoglutarate oxidation by rat liver.

had received 300 mg./kg. of AET 30 minutes before sacrifice exhibited only 12 percent inhibition of enzyme activity. A concentration of 3×10^{-3} molar AET produced 34 percent inhibition of the malic dehydrogenase activity of kidney. The in vivo effects of 300 mg./kg. of AET on the kidney enzyme were quantitatively similar to those in liver. AET, both in vitro and in vivo, produced its maximum inhibition of malate oxidation during the early stages of the reaction period and in all cases activity returned to normal within 40 minutes. Thus it appears that the reduced form of AET is the inhibitor of malic dehydrogenase since oxidation of the drug during the course of incubation resulted in reversal of enzyme inhibition.

Influence of AET on electron transport in rat liver

In view of the widespread effects of AET on the oxidizing enzymes of the citric acid cycle, it was considered advisable to study the effects of the drug on individual components of the electron transport system which is involved in many of these reactions. The results of experiments on the effects of 2×10^{-3} molar AET on the DPNH-cytochrome c reductase complex of rat liver mitochondria are presented in table II. Control activity ranged from 45 to 55 μg. of reduced DPN oxidized by mitochondria from 10 mg. of tissue per minute. The addition of AET at zero time brought about no significant change in the rate of the reaction whereas preincubation of the enzyme with the drug for 20 and 40 minutes at 38° C. produced a marked decrease of enzyme activity. Cytochrome c was a necessary constituent of the preincubation medium for maximum inhibition by the drug. Replacement of the mitochondria of the preincubation mixture with fresh tissue indicated that no inhibitor had accumulated in the incubation medium. It was found that

TABLE II

Action of AET on the DPNH-cytochrome c reductase activity of rat liver mitochondria in vitro

Expt. No.	Preincubation medium – 38° C.			Percent of control activity		
	Phosphate buffer	Cytochrome *c*	Mitochondria	Preincubation time (min.)		
				0	20	40
1	+	+	+	106	42	16
2	+	0	+	–	60	41
3	+	0	+ (replaced)†	–	112	92
4	+	+	+ (replaced)†	–	86	69
5	+	+ (replaced)*	+ (replaced)†	–	110	100
6	+	+	0	–	111	113

*Additional cytochrome *c* added before activity determination.

†Mitochondria removed by centrifugation at 10,000 × g for 10 minutes and replaced by fresh mitochondrial suspension.

incubation of AET with tissue plus cytochrome *c* followed by replacement of the tissue resulted in moderate inhibition of activity. However, the addition of fresh mitochondria and cytochrome *c* after incubation with AET completely reversed this effect. Since AET failed to react with cytochrome *c* in the absence of tissue, it may be concluded that the inhibitory action of the drug in this system is the result of partial inactivation of cytochrome *c* which takes place only in the presence of tissue and to inhibition of the reductase enzyme complex either in the presence or absence of cytochrome *c*.

It was also considered necessary to test the effect of AET on cytochrome oxidase, which is an essential component of the succinoxidase system, before ascribing its inhibitory action on succinate oxidation to a direct effect on the dehydrogenase enzyme. For these studies molar concentrations of AET ranging from 1×10^{-4} to 5×10^{-3} were incubated with rat liver homogenates. The average control Q_{O_2} for the cytochrome oxidase activity of the livers of 4 animals was 341, and 50 percent inhibition of the enzyme activity was produced by 2×10^{-3} molar AET. The administration of 200 mg./kg. of AET to rats 30 minutes before sacrifice had no effect on the cytochrome oxidase activity of the liver.

Effects of AET on the activity of oxidative enzymes in liver of various species

If the ability of AET to inhibit sulfhydryl enzymes is related to the toxicity of the compound, species differences in enzyme inhibition should be demonstrable since considerable variation exists between species with respect to their ability to tolerate the drug. In this connection the approximate LD_{50} values for AET dichloride in dogs, guinea pigs, rats, and mice are 110 mg./kg., 175 mg./kg., 325 mg./kg., and 400 mg./kg., respectively. In view of these differences, a comparison of the effects of AET on the oxidation of pyruvate, alpha-ketoglutarate, and succinate as well as cytochrome oxidase activity of the liver of these species was conducted. These results are presented in table III where values shown represent the averages for 2 to 5 animals.

A concentration of 1×10^{-3} molar AET produced 54 percent and 15 percent inhibition of the oxidation of pyruvate in rat and mouse liver, respectively, while a negligible depression was observed in dog liver. Pyruvate oxidation in guinea pig liver was refractory to inhibition by AET. The in vivo effects of 190 mg./kg. of AET administered 30 minutes before sacrifice were in general agreement with the effects in vitro.

The relationships noted above also existed in the succinic dehydrogenase system. A concentration of 1×10^{-3} molar AET caused 47 percent depression of succinate oxidation in rat liver while 33 percent and 31 percent inhibition was observed in mouse and dog liver, respectively. The only species affected in vivo by 190 mg./kg. of AET was the mouse. The in

TABLE III

Effect of AET on the oxidative carbohydrate metabolism of liver tissue in several mammalian species

		Rat	Mouse	Guinea pig	Dog
Pyruvate oxidation	Control activity*	5.6 ± 0.4	6.8 ± 0.6	1.8 ± 0.3	2.1 ± 0.2
	Inhibition in vitro†	54	15	0	5
	Inhibition in vivo	54	31	0	–
Alpha-ketoglutarate oxidation	Control activity	9.3 ± 0.8	11.0 ± 0.5	2.8 ± 0.4	5.0 ± 0.2
	Inhibition in vitro	41	0	0	0
	Inhibition in vivo	25	9	0	–
Succinate oxidation	Control activity	78 ± 2	83 ± 12		71 ± 3
	Inhibition in vitro	47	33		31
	Inhibition in vivo	5	17		–
Cytochrome oxidase	Control activity	341 ± 28	209 ± 40		147 ± 2
	Inhibition in vitro	45	11		18
	Inhibition in vivo	0	–		–

*Pyruvate and alpha-ketoglutarate oxidation activities represent μM. of substrate utilized/50 mg. of tissue/40 minutes. Succinate oxidation and cytochrome oxidase activities represent $(K)_2$.

†Inhibition represents the average of inhibitions of enzyme activity in the tissues of 2 to 5 animals.

vitro effects of AET on liver cytochrome oxidase activity again indicated that the rat was the most susceptible species studied. Thus, while these experiments reveal differences between mammalian species regarding the susceptibility of liver enzymes to inhibition by AET, they do not indicate a relationship between these effects and the toxicity of the compound.

Effects of AET on glycolysis in rat liver

Since a number of the enzymes involved in anaerobic glycolysis are sulfhydryl-dependent, a study was undertaken to determine the effects of AET on this phase of carbohydrate metabolism. It was found that normal rat liver slices formed 11.0 μg. of lactate/mg. of tissue/hour from endogenous glycogen and this value was increased 26 percent and 65 percent in the presence of 1×10^{-3} molar and 5×10^{-3} molar AET, respectively. In vivo confirmation of this effect was obtained when it was observed that the administration of AET to rats led to marked increases in blood lactate concentrations. A summary of these results is presented in figure 4 where each curve represents average values obtained with 4 animals. The maximum degree of lactacidemia occurred at one hour after the injection of AET and was almost completely reversed within 6 hours after sublethal doses. Blood pyruvate concentrations in these animals were not appreciably affected as evidenced by a maximum rise of 27 percent from a control level of 0.99 mg. percent at 1 hour after 300 mg./kg. of AET.

In an effort to localize the site of stimulation of glycolysis, 0.02 molar glucose was added to liver slices and the rate of lactate formation was determined manometrically. The control QCO_2 was 12.3 and AET concentrations of 1×10^{-3} molar and 3×10^{-4} molar had no effect on the reaction rate. Thus it appears that AET effects one of the reactions which produces glucose-6-phosphate from glycogen. This possibility was tested by measuring the rate of liberation of glucose from rat liver slices in vitro. It was found that the control rate of 10.7 mg. of glucose liberated/gram of tissue/40 minutes was not altered by concentration of AET as high as 1×10^{-3} molar. The addition of 18 mg. of glucose-1-phosphate per sample resulted in an increase of activity to 24.9 mg. of glucose/gram of tissue/40 minutes but no further change was produced by the addition of AET. These results indicate that the phosphorylase,

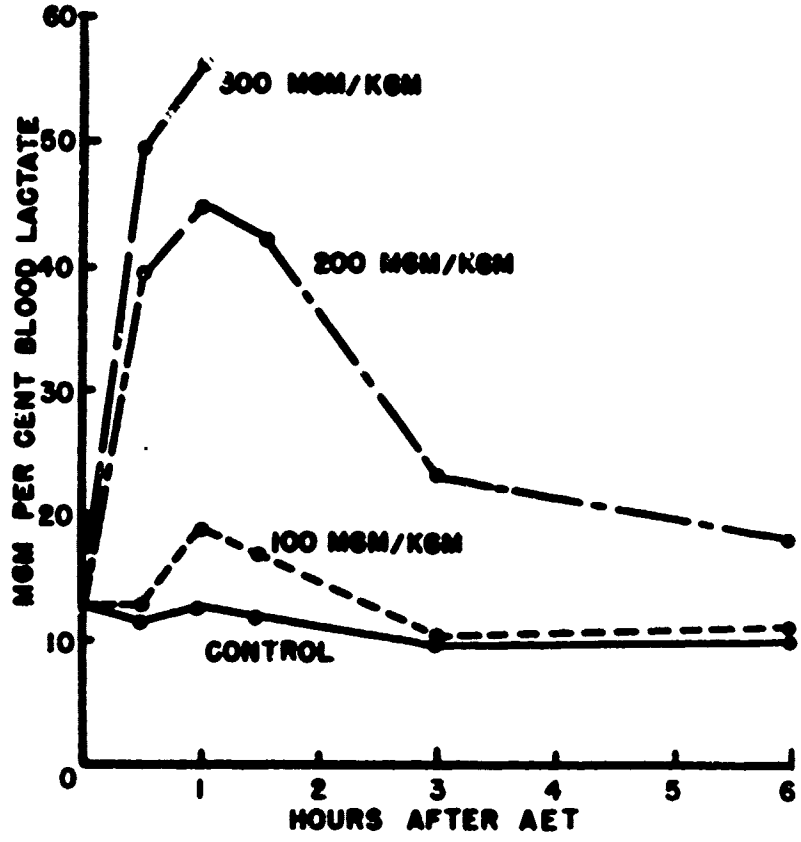

FIGURE 4

The effect of AET on blood lactate levels in rats.

phosphoglucomutase, and glucose-6-phosphatase of rat liver are not affected by AET.

Effects of AET on miscellaneous enzymes

In an effort to obtain additional information regarding the reactivity of AET with sulfhydryl enzymes, we investigated the effect of the drug in vitro on the adenosine triphosphatase activity of mouse liver, spleen, and thymus gland and on purified preparations of hexokinase and lactic dehydrogenase. The results of these experiments showed that none of these enzymes are inhibited by concentrations of AET as high as 3×10^{-3} molar.

DISCUSSION

The results of the present investigation demonstrated the ability of the radioprotective compound, AET, to inhibit a number of sulfhydryl enzyme systems which function in the oxidative phase of intermediary carbohydrate metabolism. Among the enzymes which were found to be most sensitive to the action of AET in vitro and in vivo were those which catalyze the oxidation of alpha-ketoglutaric acids by liver and kidney of rats. Evidence was obtained which indicates that these effects are due to an action by AET on the enzymes rather than on essential coenzymes involved in the reactions. It was found, however, that the activity of DPNH-cytochrome *c* reductase of rat liver mitochondria was markedly depressed in vitro by incubation with AET and, in many respects, the reductase inhibition was similar to the inhibition of alpha-ketoglutaric acid oxidation produced by the drug in rat liver and kidney. Whether DPNH-cytochrome *c* reductase is the primary locus of action of AET in these complex systems cannot, as yet, be stated with certainty since it is also distinctly possible that the sulfhydryl-bearing dehydrogenase enzymes themselves react with

the compound. It was observed that cytochrome *c* is partially inactivated by AET in the presence of tissue and this presents an additional possibility to explain the actions of AET. Although cytochrome oxidase is also inhibited by AET in vitro, it is not likely that this effect is related to the inhibition of pyruvate and alpha-ketoglutarate oxidation since the I_{50} concentrations of AET for the latter reactions were considerably lower than for cytochrome oxidase; furthermore, cytochrome oxidase of rat liver is not inhibited by AET in vivo as are the alpha-ketoglutaric acid oxidizing systems. Although AET is capable of causing marked depression of succinic and malic dehydrogenase activity in vitro, the concentrations required to produce 50 percent inhibition were two to four times greater than were required to produce an equal amount of inhibition of the alpha-ketoglutaric acid oxidizing systems. The fact that AET exerts only a moderate effect on succinic and malic dehydrogenase in vivo suggests that these enzymes are not primary sites of AET action in the intact animal. However, these results do not preclude the possibility that these enzymes are inhibited in vivo since it is possible that the procedures involved in the preparation of tissues for assay alter the relationship between the enzyme and the drug sufficiently to reverse the inhibition.

Evidence was obtained during this study which suggests that oxidation of AET by animal tissues is necessary for the inhibitory properties of the compound on DPNH-cytochrome *c* reductase as well as on pyruvate and alpha-ketoglutarate oxidation. Our data indicate that the oxidation of AET is catalyzed by appropriate enzymes which are present in rat liver, kidney, and heart but which are absent from spleen, thymus, and brain. On the basis of the results obtained in the experiments on DPNH-cytochrome *c* reductase, it does not appear that the active metabolite of AET is stable. This conclusion is based on the finding that an inhibitor does not accumulate when AET is incubated with tissue preparations.

A number of possibilities exist to explain the accumulation of lactic acid in the blood of rats treated with AET. It is likely that the enhancement of anaerobic glycolysis which was observed in liver slices contributes to the amount of lactic acid released into the blood. The inhibitory action of AET on the oxidation of the 3-carbon fragments formed from the breakdown of glucose could result in their accumulation in the animal. The fact that blood pyruvate levels failed to increase in proportion to blood lactate implies a deficiency in the ability of tissues to oxidize lactate to pyruvate. This may be the result of a reduced supply of oxygen in the tissues due to respiratory and circulatory depression (4) or to an interference with the electron transport mechanisms by inhibition of DPNH-cytochrome *c* reductase or cytochrome oxidase. In either case, cofactors and substrates would accumulate in the reduced form and thus lactate rather than pyruvate would be the end product of glucose metabolism.

The results of this investigation demonstrated that AET inhibits a number of sulfhydryl enzymes of the rat in vitro and, in the case of pyruvate and alpha-ketoglutarate oxidation, marked inhibition was produced by sublethal doses of AET in vivo. It is conceivable that these enzymatic lesions induced by AET are related to the ability of the compound to alter the function of certain organs and systems of the rat, resulting in toxic symptoms and lethality. The rapidity with which doses of AET inhibit these enzymes in vivo as well as the duration of the inhibition correlate well with the time of onset and disappearance of toxic symptoms in poisoned rats. However, the examination of a number of species other than the rat has failed to establish a correlation between the inhibition of sulfhydryl enzymes in liver and species susceptibility to the toxic effects of AET. A possible explanation for this difference, and one which has not been explored, is that the effects of AET on liver enzymes are not representative of its actions on other tissues of the body.

The results of the present investigation are of additional interest in connection with the mechanism by which AET protects animals against radiation lethality. Bacq and Herve (30) have suggested that protective agents may combine with radiosensitive constituents of cells to form radioresistant complexes which undergo dissociation after a short period of time. In this regard it appears significant

that maximum inhibition of alpha-ketoglutaric acid oxidizing enzymes occurred during the first hour after injection of protective doses of AET, which is the time during which maximum radioprotection is obtained. Although none of the enzymes which were inhibited by AET in this study have been found to be inactivated by irradiation of animals (31), it is possible that more radiosensitive enzymes which are protected by AET may yet be discovered. These experiments demonstrate the ability of AET to react with sulfhydryl-containing cellular constituents and thus provide a basis for additional studies on enzymes which may be affected by ionizing radiation, especially in radiosensitive tissues.

SUMMARY

1. A molar concentration of 3×10^{-3} AET produced no change in the endogenous respiration of rat spleen, thymus gland, and liver slices while the oxidation of succinate and pyruvate by the latter tissue was markedly depressed. The uptake of pyruvate by thymus gland slices was increased 73 percent in the presence of 2×10^{-3} molar AET.

2. The oxidation of pyruvate plus fumarate by rat liver, kidney, heart, and brain homogenates was inhibited by AET in vitro while a dose of 300 mg./kg. given 30 minutes before sacrifice failed to cause an effect in this system. However, AET at a dosage level of 200 mg./kg. reduced the citrate accumulation in rat kidney, liver, and heart following the administration of fluoroacetate.

3. The oxidation of pyruvate by rat liver and kidney homogenates was 50 percent inhibited by 4.3×10^{-4} molar AET and 3.1×10^{-4} molar AET, respectively. Heart tissue was less susceptible to inhibition while brain was completely refractory. Alpha-ketoglutarate oxidation by rat liver was 50 percent inhibited by 6.6×10^{-4} molar AET. Both enzymes in liver and kidney tissue were markedly inhibited in vivo following sublethal and lethal doses of AET. Maximum inhibition occurred 30 minutes after injection and was completely reversed within 3 hours after a sublethal dose of the compound. Inhibition of alpha-ketoglutaric acid oxidation by AET was not produced through an action on DPN, cocarboxylase, or coenzyme A.

4. The development of the inhibition of alpha-ketoglutaric acid oxidation in vitro was progressive with incubation time and accompanied oxidation of AET by the tissue. Rat liver and kidney oxidized the greatest amount of AET while heart muscle and brain were less active.

5. Succinic dehydrogenase, malic dehydrogenase, and cytochrome oxidase activity of rat liver were 50 percent inhibited by 2×10^{-3} molar AET in vitro. Malic dehydrogenase and cytochrome oxidase inhibition was partially reversed during the latter stages of incubation while depression of succinic dehydrogenase activity remained constant. The malic dehydrogenase and cytochrome oxidase activities of rat liver were not appreciably altered in vivo by AET. A dose of 380 mg./kg. of AET produced 24 percent inhibition of rat liver succinic dehydrogenase activity 30 minutes after injection.

6. DPNH-cytochrome *c* reductase activity of rat liver mitochondria was inhibited 84 percent following a 40-minute preincubation with AET at 38° C. Approximately one-third of the inhibition was attributable to an action on cytochrome *c* while the remaining effect was due to an action on the reductase complex. The presence of tissue was necessary for the effect on cytochrome *c*.

7. Liver enzymes in the rat which catalyze the oxidation of pyruvate, alpha-ketoglutarate, succinate, and cytochrome *c* were more susceptible to inhibition by AET in vitro and in vivo than the same enzymes of the mouse, guinea pig, and dog.

8. Anaerobic glycolysis in rat liver slices were stimulated by AET. This effect was not mediated through an action on the metabolic reactions involved in glucose-6-phosphate metabolism nor was any effect produced on rat liver phosphorylase or phosphoglucomutase activity. The administration of AET to rats caused increases in blood lactate concentrations in proportion to the dose of the compound employed.

9. AET at concentrations as high as 3×10^{-3} molar failed to inhibit the adenosine triphosphatase activity of several mouse tissues. Furthermore, this concentration of AET produced no inhibition of purified hexokinase and lactic dehydrogenase activity even after preincubation with the enzymes at 38° C. for 40 minutes.

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